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THE MODULATORY ROLE PLAYED BY TNF-α AND IL-1β IN THE INFLAMMATORY RESPONSES INDUCED BY CARRAGEENAN IN THE MOUSE MODEL OF PLEURISY

Tânia S. Fröde,[1] Glória E. P. Souza,[2] João B. Calixto[1]

We describe here the modulation caused by intrapleural (i.pl.) injection of the cytokines TNF-α and IL-1β and their specific antibodies in the early (4 h) and late (48 h) inflammatory responses caused by injection of carrageenan (Cg) into the mouse pleural cavity. The antibodies against TNF-α and IL-1β, when injected 30 min prior to Cg, reduced, in a graded and significant manner, both exudation and cell migration in the early (4 h) phase, while they potentiated or had no effect in the late (48 h) phase of Cg response. The natural IL-1 receptor antagonist IL-1RA, given 30 min prior to Cg, reduced the exudation by about 50% and abolished the total and differential cell migration in the early (4 h) and late (48 h) phases of the Cg responses. The i.pl. injection of TNF-α or IL-1β, 5 min prior to Cg, caused graded increase in the exudation of the early (4 h) and late (48 h) phases of the Cg-induced inflammatory responses. In contrast, these treatments markedly reduced the total and differential cell migration at 4 h, while having little or no effect on the late (48 h) phase of the Cg pleurisy. These findings extend previous results and demonstrate that the pro-inflammatory cytokines TNF-α and IL-1β have a critical role in controlling both cell migration and exudation caused by injection of Cg in the mouse pleural cavity. Together, these findings may be relevant to the understanding of the mechanisms involved in airway inflammatory responses.

Airway inflammation has a critical role in the pathogenesis of asthma. This disease is characterized by chronic inflammation with leukocyte infiltrates in the bronchial tissue, excessive mucus production, and pulmonary hyperactivity, which involve both airway and vascular smooth muscles.[1] This multifactorial disease involves the interplay of diverse chemical mediators and multicellular inflammatory events. There is now a substantial amount of evidence supporting the notion that inducible cell-derived proteins, such as cytokines and chemokines, are important mediators released during inflammatory responses, notably in asthma exacerbation or perpetuation of airway inflammation.[2–4] Thus, pro-inflammatory cytokines such as TNF-α and IL-1β, released in the lung mainly by alveolar macrophages, play a dominant role in chronic airway inflammation.[5,6] Apart from this, the pro-inflammatory products, including the chemotatic and/or inflammatory cytokines produced from T lymphocytes, among other actions, are known to control cell functions such as cell growth, immunity, differentiation and tecidual repair.[7]

In previous studies,[8,9] it has been shown that the mouse pleural inflammatory response induced by carrageenan (Cg) constitutes an interesting experimental model, characterized by two distinct phases: an early (4 h) phase characterized by the release of chemical mediators such as histamine, bradykinin and prostaglandins, and a late (48 h) phase which involves mainly PAF and leukotrienes. Although the mouse pleural inflammation induced by Cg does not fit the complete criteria in terms of airway inflammation, as it does for instance in the case of the asthma model, this model shows some interesting and specific characteristics which may render it useful to our understanding of the role played by inflammatory mediators and the

From the [1]Department of Pharmacology, Center of Biological Sciences, Universidade Federal de Santa Catarina 88015-420, Florianópolis, SC, Brazil, and [2]Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, USP, 140409-903, Ribeirão Preto, SP, Brazil

Correspondence to: João B. Calixto, Department of Pharmacology, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Rua Ferreira Lima, 82, 88015-420, Florianópolis, SC, Brazil. E-mail: calixto@farmaco.ufsc.br

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mechanisms underlying their actions in airway inflammatory responses, and, mainly, to the development of new potential anti-inflammatory drugs for the treatment of airway diseases.

However, to date no data exist to show whether or not the pro-inflammatory cytokines exert a relevant modulation of the phases of Cg-induced mouse pleural inflammation. Therefore, in the present study we sought to investigate, by use of specific antibodies and by use of the IL-1β receptor antagonists, the possible modulatory actions exerted by TNF-α and IL-1β in the early (4 h) and late (48 h) phases of the pleural inflammatory response following i.pl. injection of Cg in mice.

RESULTS

The intrapleural (i.pl.) injection of the anti-TNF-α monoclonal antibody (mAb) (5–50 ng/cav.), given 30 min prior to Cg, produced a significant decrease in both exudation (23 ± 12; 41 ± 10 and 37 ± 12% at 5, 10 and 50 ng/cav., respectively) and total cell migration (56 ± 8; 48 ± 12 and 63 ± 7% at 5, 10 and 50 ng/cav., respectively) when assessed in the early (4 h) phase of Cg-induced pleurisy ($P<0.01$) (Fig. 1A, C). Likewise, the differential influx of neutrophils, but not that of mononuclear cells, was also inhibited by anti-TNF-α ($P<0.01$) (Fig. 1B). In contrast, when assessed in the late (48 h) phase of the Cg inflammatory response, the anti-TNF-α mAb, at doses of 5 to 50 ng/cav., elevated cell migration by 75 ± 12 and 40 ± 7% and exudation by 86 ± 2 and 59 ± 7% in response to i.pl. injection of Cg ($P<0.01$) (results not shown).

Figure 2 A shows that i.pl. injection of the anti-IL-1β (5, 50 and 100 ng/cav.) significantly reduced, by 52 ± 14, 69 ± 10 and 66 ± 3%, the total cell influx in relation to the early (4 h) phase of the pleurisy induced by Cg. The same doses of anti-IL-1β also significantly inhibited the neutrophil and mononuclear cell influx in response to i.pl. injection of Cg ($P<0.01$) (Fig. 2B). However, only the high dose of anti-IL-1β antibody (100 ng/cav.) significantly inhibited (66 ± 10%) the exudation caused by i.pl. injection of Cg ($P<0.01$) (Fig. 2C). When assessed in the second (48 h) phase of Cg response, the anti-IL-1β antibody, at doses of 5 and 50 ng/cav., as reported for anti-TNF-α antibody, markedly increased the influx of leukocytes (75 ± 19 and 67 ± 11%) and exudation (65 ± 5 and 49 ± 6·5%), respectively (results not shown).

The i.pl. injection of the natural IL-1 receptor antagonist IL-1RA (1, 5 and 10 μg/cav.), 30 min prior to Cg, produced, at all doses, marked total leukocyte influx caused by Cg (68 ± 6, 55 ± 6 and 71 ± 7%, respectively). The differential cell influx (neutrophil and mononuclear cells) was significantly reduced by IL-1RA in the early (4 h) phase of the Cg response

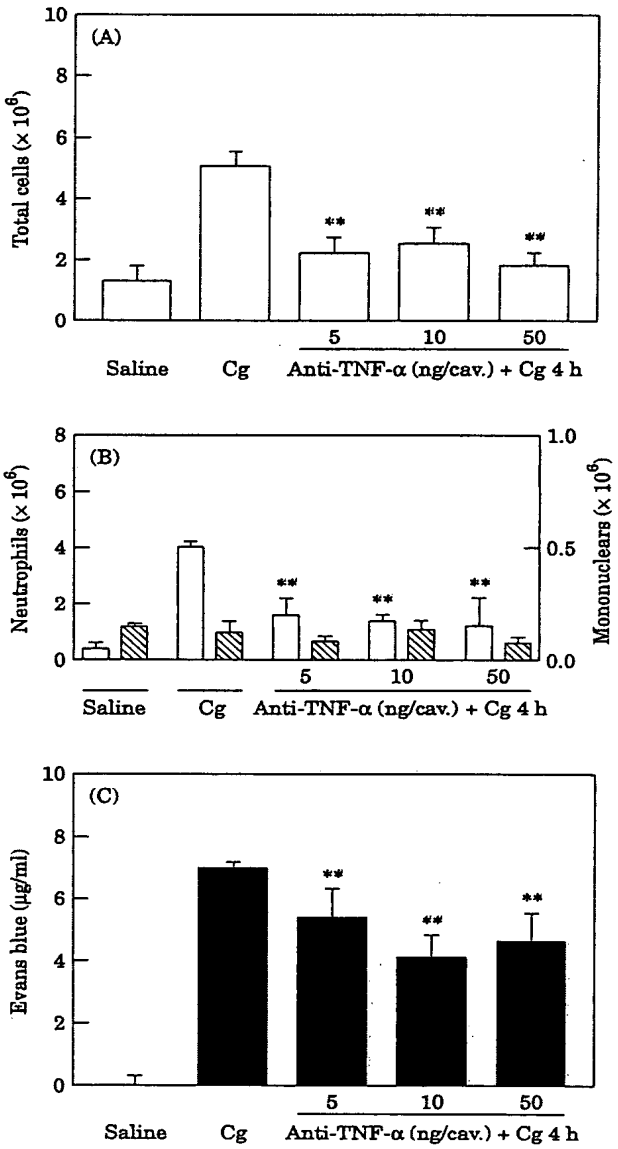

Figure 1. Effect of intrapleural administration of anti-TNF-α mAb on the early (4 h) phase of mouse pleurisy induced by carrageenan (1% per pleural cavity).

Anti-TNF-α mAb was injected directly into the pleural cavity 30 min prior to Cg. (A) total cells; (B) neutrophil (□) and mononuclear (▨) cells; and (C) Evans blue content (exudation). Each group represents the mean of six animals and the vertical bars represent the SEM. Significantly different (**$P<0.01$) when compared with carrageenan-treated group.

($P<0.01$) (Figs 3A and B). Similarly, IL-1RA (5 and 10 μg/cav.) reduced, by 34 ± 6 and 55 ± 5%, respectively, the exudation caused by Cg ($P<0.01$) (Fig. 3C). The IL-1RA, at doses ten times higher (75 and 100 μg/cav.), significantly inhibited the total leukocyte influx (30 ± 6 and 33 ± 7%) and exudation (22 ± 11 and

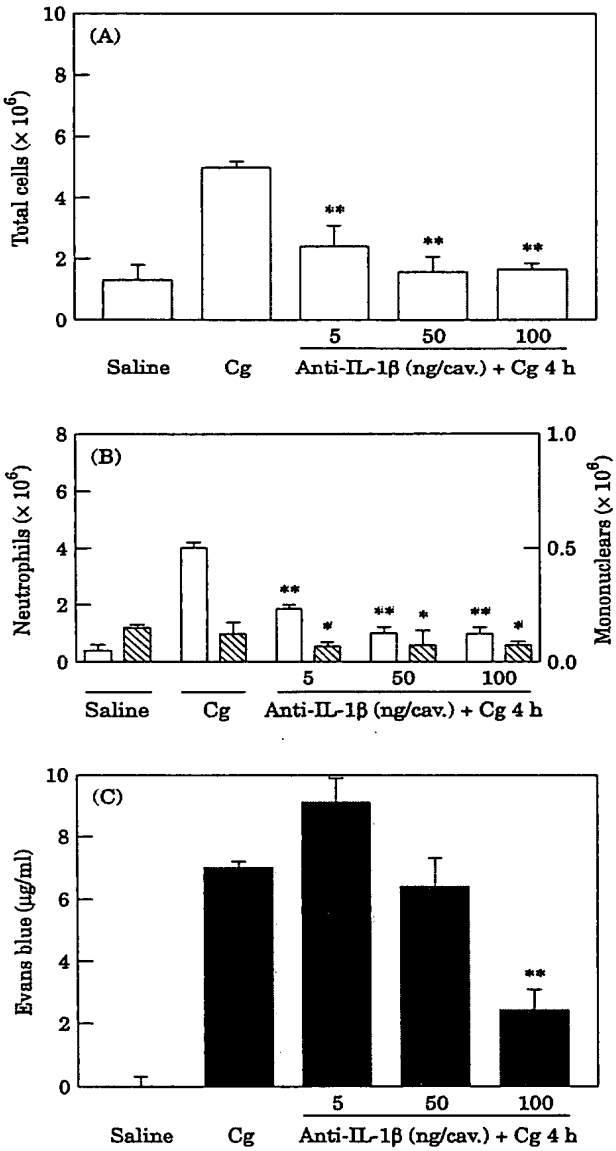

Figure 2. Effect of intrapleural administration of anti-IL-1β mAb on the early (4 h) phase of mouse pleurisy induced by carrageenan (1% per pleural cavity).

Anti-ILβ mAb was injected directly into the pleural cavity 30 min prior to Cg. (A) total cells; (B) neutrophil (□) and mononuclear (▨) cells; and (C) Evans blue content (exudation). Each group represents the mean of six animals and the vertical bars represent the SEM. Significantly different (**$P<0.01$) when compared with carrageenan-treated group.

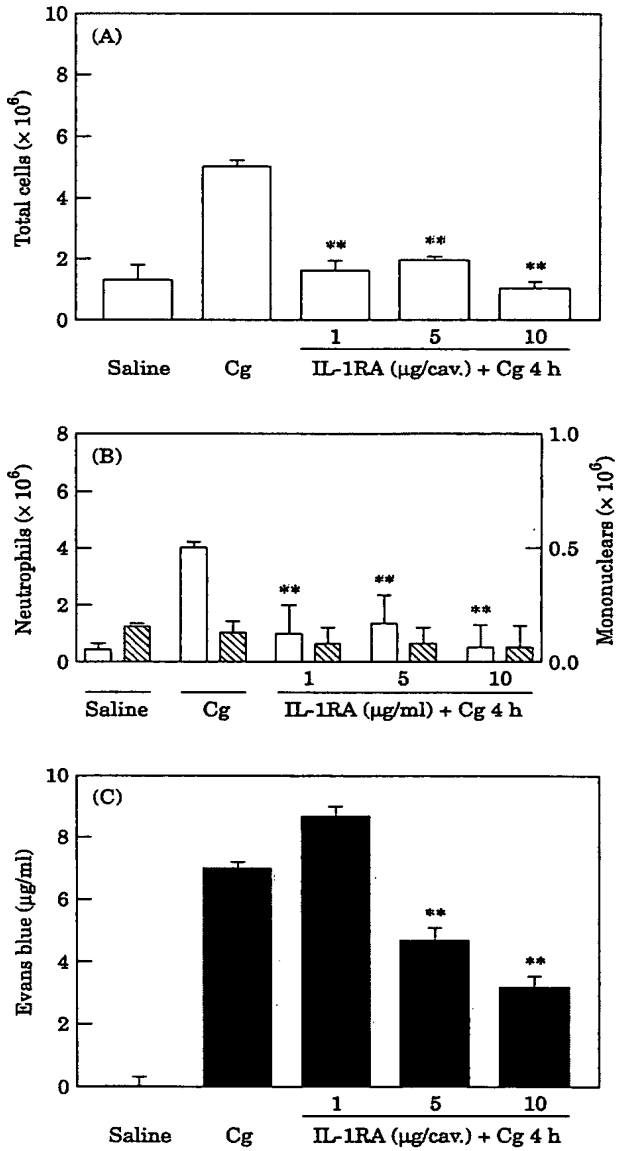

Figure 3. Effect of intrapleural administration of IL-1 receptor antagonist IL-1RA on the early (4 h) phase of mouse pleurisy induced by carrageenan (1% per pleural cavity).

IL-1RA was injected directly into the pleural cavity 30 min prior to Cg. (A) total cells; (B) neutrophil (□) and mononuclear (▨) cells; and (C) Evans blue content (exudation). Each group represents the mean of six animals and the vertical bars represent the SEM. Significantly different (**$P<0.01$) when compared with carrageenan-treated group.

66 ± 6%, respectively) of Cg late (48 h) phase response (results not shown).

Figure 4(A and B) shows that i.pl. injections of TNF-α (0.01, 0.1 and 5 ng/cav.), given 5 min prior, significantly reduced the total cell influx (59 ± 6, 57 ± 6 and 64 ± 2%) as well as the differential migration of neutrophils and mononuclear cells ($P<0.01$). On the other hand, the same doses of TNF-α increased by 244 ± 14, 286 ± 23 and 343 ± 29%, respectively, the exudation caused by Cg when assessed at 4 h ($P<0.01$) (Fig. 4C). Higher doses of TNF-α (0.5, 5 and 50 ng/cav.) produced a remarkable increase (135 ± 10, 198 ± 10 and 409 ± 7%) in the Cg-induced pleural exudation 48 h later.

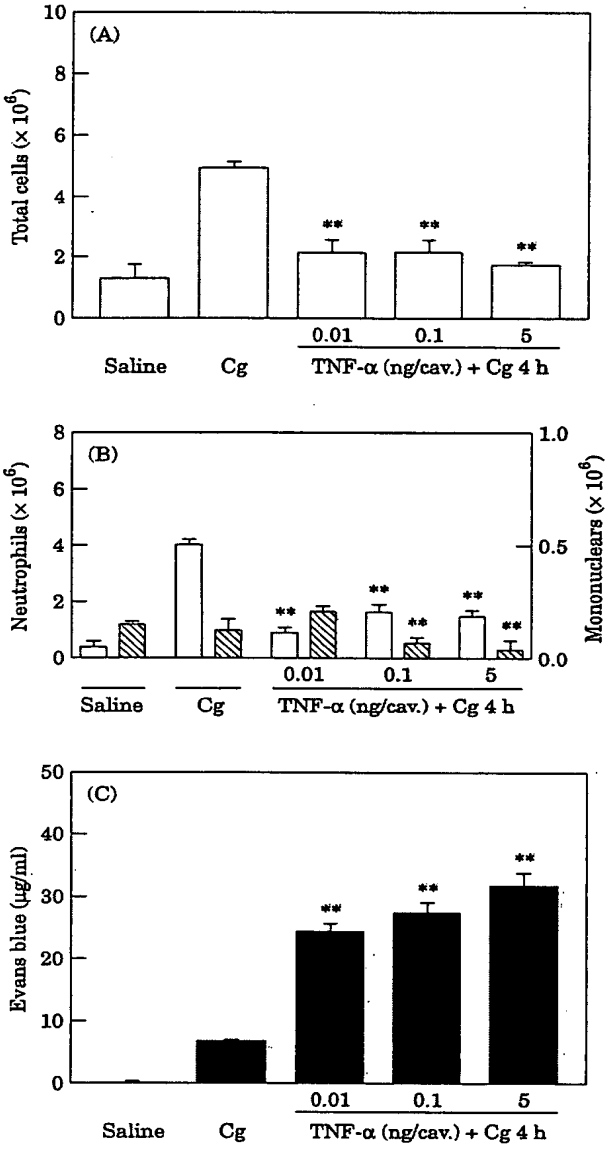

Figure 4. Effect of intrapleural administration of TNF-α on the early (4 h) phase of mouse pleurisy induced by carrageenan (1% per pleural cavity).

TNF-α was injected directly into the pleural cavity 30 min prior to Cg. (A) total cells; (B) neutrophil (□) and mononuclear (▨) cells; and (C) Evans blue content (exudation). Each group represents the mean of six animals and the vertical bars represent the SEM. Significantly different (**$P<0.01$) when compared with carrageenan-treated group.

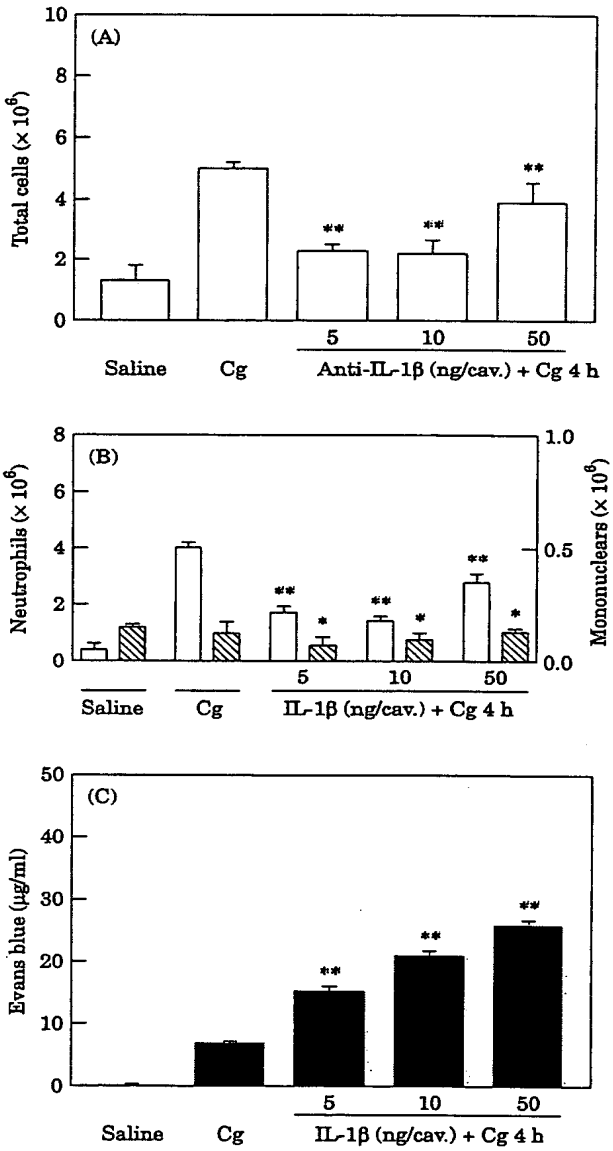

Figure 5. Effect of intrapleural administration of IL-1β on the early (4 h) phase of mouse pleurisy induced by carrageenan (1% per pleural cavity).

IL-1β was injected directly into the pleural cavity 30 min prior to Cg. (A) total cells; (B) neutrophil (□) and mononuclear (▨) cells; and (C) Evans blue content (exudation). Each group represents the mean of six animals and the vertical bars represent the SEM. Significantly different (**$P<0.01$) when compared with carrageenan-treated group.

Similar to TNF-α, the i.pl. injection of IL-1β (5, 10 and 50 ng/ml), prior to Cg, reduced by 57 ± 4, 59 ± 7 and 45 ± 10%, respectively, the total influx of leukocyte cells and both neutrophil and mononuclear cell migration in response to i.pl. injection of Cg when assessed at 4 h (Figs 5A and B) ($P<0.01$). The exudation caused by Cg was significantly increased by IL-1β (115 ± 10, 200 ± 12 and 270 ± 9%, respectively) ($P<0.01$) (Fig. 5C). In the late (48 h) phase of the Cg inflammatory response, IL-1β (0.01 and 0.05 ng/cav) significantly increased the total cell influx (42 ± 11 and 56 ± 15%) and exudation (84 ± 18 and 310 ± 20%) caused by i.pl. injection of Cg (results not shown).

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DISCUSSION

We have shown here, by the use of monoclonal antibodies for both TNF-α and IL-1β and also by the use of selective receptor antagonist for IL-1β, IL-1RA, clear evidence indicating the involvement of both pro-inflammatory cytokines in the mouse pleural inflammatory response induced by Cg. This modulatory effect caused by cytokines TNF-α and IL-1β, however, was more evident in relation to the early (4 h) phase than to the late (48 h) response of the Cg-induced pleural inflammation in mice. We have also shown here that i.pl. injection of microgram doses of IL-1RA, prior to i.pl. administration of Cg into the pleural space, consistently inhibited the total and differential (neutrophil and mononuclear) cell numbers in the pleural cavity, as well as the exudation 4 h after the irritant injection. At higher doses, IL-1RA also significantly blocked both cell migrations and exudation in response to Cg. These results are in agreement with that from Perretti *et al.*[10] who reported that IL-1RA inhibited the neutrophil migration induced by IL-1β in the mouse air pouch.

Another new and interesting result of the present study was the demonstration that the monoclonal antibodies against IL-1β and TNF-α markedly blocked the total and differential leukocyte influx in the early (4 h) phase of the Cg-induced lung inflammatory response. Furthermore, the anti-TNF-α antibody, and, to a lesser extent the anti-IL-1β, also consistently prevented the fluid leakage caused by Cg in the mouse model of pleurisy. In contrast, when assessed in the late (48 h) phase, both the anti-TNF-α and anti-IL-1β antibodies produced pronounced enhancement of both total and differential cell migration to the pleural cavity and the exudation, thus suggesting that distinct mechanisms might be involved in the two phases of Cg-mediated mouse pleural inflammation. This is consistent with the previous results suggesting that different inflammatory mediators, including certain cytokines, are involved in each phase of the rat carrageenan-induced pleural inflammatory response.[11,12] We cannot completely discard the hypothesis that these differences may be associated to the half-life of the antibody, because the antibody could be inactivated by seric enzymes while the inflammatory stimulus remained.

The injection of both TNF-α and IL-1β directly into the pleural space prior to i.pl. injection of Cg, analyzed 4 h after, produced a pronounced and dose-dependent potentiation of Cg-induced exudation (maximal potentiation of about 350%), associated in all cases with significant, though not dose-dependent, inhibition of both total and differential leukocyte migrations to the pleural cavity. These results are, to some degree, in contradiction with those reported for animals treated with both the anti-TNF-α and IL-1β monoclonal antibodies. However, a plausible explanation for such discrepant findings is that an excess of IL-1β or TNF-α may activate inhibitory systems to reduce cell migration and also stimulatory systems to increase exudation, demonstrating the attempt of the organism to prevent tissue injury.

IL-1 interacts with at least two types of receptors, Type I and Type II IL-1 R. Type I is predominantly found in T lymphocytes and fibroblasts, while Type II is expressed in B lymphocytes, monocytes and neutrophils. However, several studies have shown the presence of Type I receptors on neutrophils and endothelial cells.[13, 14] It has also been demonstrated that IL-1 signalling occurs exclusively via Type I receptors, while Type II is an inhibitory receptor, like the IL-1 receptor antagonist IL-1RA (for review, see Brooks and Mizel, 1994).[15] Apart from that, the IL-1RA has been previously shown to be effective in inhibiting LPS-or IL-1-induced acute pulmonary inflammation,[16] fever, expression of ICAM and ELAM and IL-6 release by endothelial cells,[17] showing the importance of Type I receptors on endothelial cells in mediator release and cell migration.

There is now a considerable amount of evidence suggesting that TNF-α exerts a pivotal role in the lung inflammatory responses challenged by several microbes, including *Strepetoccus pneumoniae, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Mycobacterium tuberculosis*.[18–21] Recently, Smith *et al.*[22] have provided more conclusive data by using transgenic mice that express a soluble TNF-α inhibitor, affirming that TNF-α contributes to the lung defences against microbes by acting at pulmonary and/or extra-pulmonary sites, depending on the nature of the inflammatory stimulus.

The mechanisms, however, by which TNFα and IL-1β exert their modulatory action on Cg-mediated pleural inflammatory response in mice remain unknown. It has been reported previously, by Amrani *et al.*,[23] that both cytokines, when tested in human airway smooth muscle cells, enhance thrombine- and bradykinin-induced alteration of calcium responsiveness. Furthermore, these cytokines have been found effective in inducing airway hyperactivity and neutrophilia in normal human subjects.[24] Both TNF-α and IL-1β treatments of rats are capable of increasing the expression of cyclo-oxygenase Type 2 and phospholipase A_2 levels in airway smooth muscle cells.[25] Most of the mediated effects caused by TNF-α and IL-1β have been demonstrated to be prevented by inhibitor of protein synthesis cycloheximide and by dexamethasone.[23,25] Such mechanisms could account, at least in part, for the altered responsiveness reported here for Cg-mediated inflammatory response in the mouse model of pleurisy. Additional studies are required to

clarify the mechanisms responsible for the modulatory response of Cg-induced inflammation in the mouse pleural space.

In summary, we have demonstrated that both TNF-α and IL-1β exert critical modulatory action in the acute pleural inflammatory reaction caused by Cg when assessed in the mouse model of pleurisy. However, there are marked differences among the actions of IL-1β and TNF-α in the different phases of the pleural inflammatory response caused by Cg, suggesting that distinct mechanisms are involved in each. As cytokines such as TNF-α and IL-1β are known to exert a critical role in most inflammatory states, particularly in airway diseases,[26–31] the results of the current study reveal that the mouse inflammatory response caused by Cg constitutes a useful and interesting model for the study of new anti-inflammatory drugs, especially for the management of respiratory diseases.

MATERIAL AND METHODS

Animals and reagents

Non-fasted adult Swiss mice of both sexes (18–25 g), aged 2 months, were used throughout the experiments. The animals were kept in an environmentally controlled room temperature (21 ± 2°C) under a light/dark cycle of 12 h and were allowed free access to food and water. Animals were managed using the principles and guidelines for the care of laboratory animals according to Zimmermann.[32]

In preliminary experiments, in order to select the ideal dose of Cg, different groups of animals (n=5 in each group, results not shown) received 0.1 ml of sterile saline containing different concentrations of Cg (0.5, 1 and 2%) injected into the pleural space. Animals were killed 4 or 48 h following i.pl. Cg injection, and the exudation (by measuring Evans blue dye extravasation into the pleural cavity) and total cell migration (total leukocytes, neutrophils and mononuclear cells) were evaluated (see below). The dose of 0.1 ml of Cg (1% per pleural cavity) produced the best results and was selected for future experiments.

Experimental procedures

The experiments were conducted according to the procedures described previously by Henriques *et al.*[9] and Saleh *et al.*[8] Briefly, the mouse pleurisy was induced by a single i.pl. injection of 0.1 ml of sterile saline containing Cg (1%). As the inflammatory response caused by Cg in the pleural space of the mice exhibits a biphasic response, peaking at 4 h, characterized primarily by neutrophils, and at 48 h due mainly to mononuclear cells,[8] both interval-points were studied.

In preliminary experiments (n=4 to 10, results not shown), different intervals of pre-treatment and different doses of pro-inflammatory cytokines (TNF-α, IL-1β) were evaluated. Based on these preliminary experiments, the dose of pro-inflammatory cytokines and the respective antibody anti-cytokines from 0.01 to 50 ng/cav. and 5 to 100 ng/ml, respectively, or a natural receptor antagonist of IL-1β (IL-1RA: 1–10 μg/cav.), were selected, and the period of pre-treatment of 5 to 30 min was chosen.

On the day of the experiments, animals were lightly anaesthetized with ether, and 0.1 ml of different doses of TNF-α (0.01–5 ng/cav.) or IL-1β (5–50 ng/cav.), the antibody against these cytokines anti-TNF-α mAb (5–50 ng/cav.), anti-IL-1β mAb (5–50 ng/cav.), as well as IL-1RA (1–10 μg/cav.), a natural receptor antagonist of IL-1β, were administered into the pleural space 5 to 30 min before Cg injection. The inflammatory parameters were analyzed 4 or 48 h after pleurisy induction. Control animals received the same volume (0.1 ml) of sterile buffered-saline solution in the pleural cavity.

Animals were killed with an overdose of ether. Immediately after opening the thorax, the pleural cavity was washed with 1 ml of sterile PBS containing heparin (20 IU/ml) and the volume was collected with automatic pipettes. All animals were injected 60 min previously with a solution of Evans blue dye (25 mg/kg, 0.2 ml, i.v.) in order to evaluate the degree of exudation in the pleural space.[8] The total leukocyte counts were performed on an automatic counting machine. Cytospin preparations of pleural washing were stained with May–Grunwald–Giemsa for the differential count of the leukocytes, which was performed under immersion objective. A sample of the collected fluid (500 μl) from the pleural cavity was separated and stocked in the freezer (−20°C) to determine further the concentration of Evans blue dye. To this end, on the day of the experiments a batch of samples was thawed at room temperature, and the amount of the dye was estimated by colorimetry (Compu-Espectro Spectrometer, Brazil) at 600 nm by interpolation from a standard curve of Evans blue dye in the range of 0.01 to 50 μg/ml.

Drugs

The following drugs were used: carrageenan lambda IV (degree IV) (Sigma Chemical Co., St. Louis, MO, USA), TNF-α, IL-1β, IL-1RA, anti-TNF-α, anti-IL-1β (R & D Systems, Inc., Minneapolis, MN, USA), Evans blue dye, (Merck, Brazil), phosphate buffered saline (PBS: pH 7.6: composition mmol: NaCl 137, KCl 2.7 and phosphate buffer salts 10), purchased from Sigma Chemical Company. All cytokines were prepared daily by diluting them in PBS solution. They were kept in siliconised plastic tubes and maintained at −70°C. On the day of the experiments, the drugs were thawed and diluted to the desired concentration with PBS solution.

Statistics

Statistical differences in all experimental groups were determined by the use of one-way ANOVA test, followed by Dunnett's post-test or Student's unpaired t-test, when indicated. P values shown in the legends and in the text represent the indication of Dunnett's test or Student's unpaired t-test. Statistically significant differences were reported at $P<0.05$ or less.

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